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Original scientific paper

Development and validation of a solid phase extraction-HPLC method for the determination of carbamazepine and its metabolites, carbamazepine epoxide and carbamazepine *trans*-diol, in plasma

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Abstract: A solid phase extraction-HPLC method has been developed and validated for the rapid analysis of carbamazepine and its two metabolites, carbamazepine epoxide and carbamazepine *trans*-diol, in human plasma. The analysis was performed using a C18 Bakerbond-BDC analytical column (250 mm×4.6 mm i.d., particle size 5 μm). The optimal conditions for the separation were established with the mobile phase acetonitrile – 10 mM phosphate buffer, pH 7.0 (30:70, v/v) at a flow rate of 1.5 mL min^{-1} and temperature of 35 °C, with UV detection at 210 nm. The total run time was about 8 minutes. The SPE procedure for the extraction of the analytes from a plasma sample was developed using Oasis HLB cartridges and subsequently, the eluate was injected into the HPLC system for analysis. Afterwards, the SPE-HPLC method was subjected to validation. Linearity was obtained over the concentration range of 0.2–25 μg mL^{-1} for carbamazepine, carbamazepine epoxide and carbamazepine *trans*-diol, with correlation coefficients higher than 0.995. The method showed good intra-day and inter-day precision with a relative standard deviation below 7.96 %, while the accuracy ranged from 92.09 to 108.5 % for all analytes. Finally, the method was successfully applied to the analysis of the plasma samples of epileptic patients in mono- and polytherapy.

Keywords: human plasma; carbamazepine; carbamazepine epoxide; carbamazepine *trans*-diol; solid phase extraction.

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INTRODUCTION

Carbamazepine (CBZ, Fig. 1a) is a tricyclic lipophilic compound used in the treatment of epilepsy, trigeminal neuralgia and bipolar disorders.[1–3] A CBZ plasma concentration ranging from 4 to 12 μg mL^{-1} is associated with seizure control.[4,5] CBZ is a strong inducer of microsomal enzymes (cytochrome P450 in liver) which can quicken its own metabolism and those of co-administered drugs.[5] Hence, polytherapy may be associated with drug interactions and undesired toxicity.

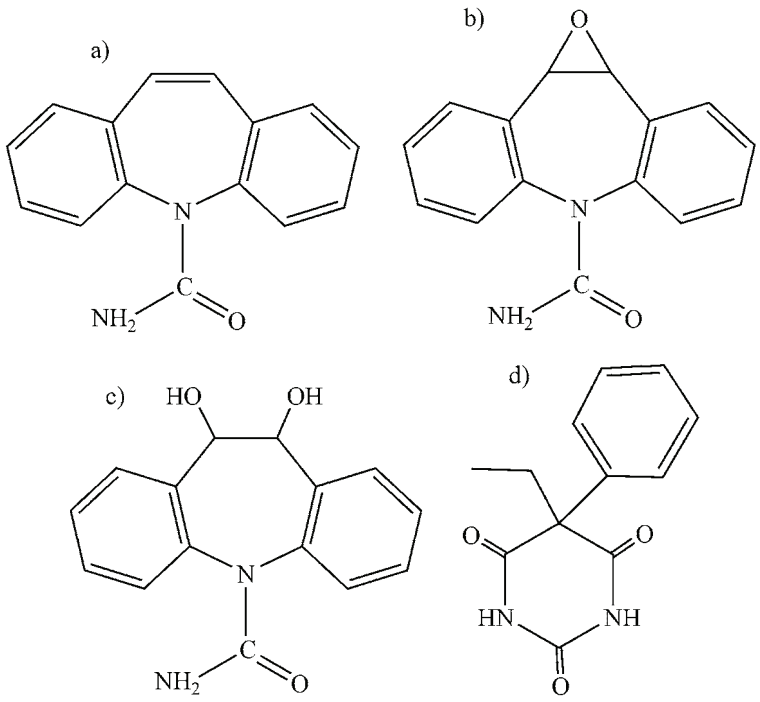

Fig. 1. Structures of carbamazepine, CBZ (a), carbamazepine epoxide, CBZ-E (b), carbamazepine *trans*-diol, CBZ-DIOH (c) and the internal standard phenobarbital (d).

Carbamazepine epoxide (CBZ-E, Fig. 1b) is the most important among its 33 metabolites, because CBZ-E exerts pharmacological activity as does its parent compound.[6] Afterwards, CBZ-E is mainly metabolized by epoxide hydrolases to carbamazepine *trans*-diol (CBZ-DIOH, Fig. 1c). CBZ-DIOH is fairly conjugated with glucuronic acid and excreted in urine.[7] Therefore, regular measurement of the plasma levels of CBZ and its metabolites and dosage adjustment are necessary for patients undergoing chronic treatment with CBZ.

Commercially immunoassays are available for the determination of the concentration of CBZ. However, CBZ-E cannot be routinely monitored using immu-

noassays and therefore measurement of both CBZ and CBZ-E requires chromatographic analysis.[8]

According to a literature survey, HPLC and HPLC-MS methods have been published for the quantitative analysis of CBZ, its metabolites and other medications in human plasma. The HPLC-MS techniques are not widely used because the required expensive equipment is not available in most clinical laboratories. Prior to HPLC analysis, human plasma samples were subjected to protein precipitation, liquid–liquid extraction[9–15], stir bar-sorptive extraction[16] or solid phase extraction (SPE).[7,17–21] Unfortunately, these methods[7,17–21] are time-consuming on account of eluate evaporation and subsequent reconstitution in comparison with the SPE-HPLC method proposed herein. After the employed SPE procedure, the eluate is injected into the HPLC system without performing evaporation and reconstitution steps. Since the total time for sample preparation is shorter than 10 min, the proposed SPE-HPLC method is advantageous over the previously published SPE-HPLC methods[7,17–21] in routine application.

An automated and sensitive SPE-HPLC method for analysis of CBZ, CBZ-E and CBZ-DIOH was found. This method was applied to the analysis of plasma samples obtained from rats treated with CBZ,[22] but the applicability of the method to samples obtained from epileptic patients was not examined. According to the chromatogram of spiked drug-free plasma, interfering substances from human plasma were not completely separated from peaks of the analytes and internal standard.[22] Considering the proposed SPE-HPLC method, the total chromategraphic run time was about 8 min with excellent peak shapes and good resolution between the investigated compounds and interfering plasma substances. Furthermore, expenses and the overall time of therapeutic drug monitoring are reduced, which is important in the individualization of therapy of patients undergoing chronic treatment with CBZ.

SPE-HPLC method was subjected to validation according to US Food and Drug Administration (FDA)[23] and International Conference on Harmonisation (ICH)[24] guidelines. It fulfills the validation criteria in every segment of validation. Finally, the developed method was successfully applied to routine analysis of plasma samples of epileptic patients under both mono- and polytherapy. It could be used for cost-effective therapeutic drug monitoring of CBZ due to its appropriate sensitivity and selectivity.

EXPERIMENTAL

Chemicals and reagents

CBZ and internal standard phenobarbital (IS, Fig. 1d) as solid standard compounds were kindly provided by pharmaceutical company Galenika (Belgrade, Serbia). CBZ-E and CBZ--DIOH as solid standard compounds were kindly provided by pharmaceutical company Novartis Pharma (Basel, Switzerland). Acetonitrile and methanol HPLC gradient grade were purchased from Avantor Performance Materials (Deventer, The Netherlands). Sodium hydro-

xide and sodium dihydrogen phosphate suitable for HPLC were obtained from Merck (Darmstadt, Germany).

Instrumentation and materials

HPLC analysis was performed with an Agilent Technologies 1200 (Wilmington, DE, USA) chromatographic system equipped with on-line degasser, binary pump, column oven and photo diode array detector. Sample injection of 10 μL was performed using an Agilent 1200 Series high performance autosampler G1367B. Water for chromatography was obtained from a Smart 2 Pure (TKA, Niederelbert, Germany) purification system. Before use, the mobile phase was degassed and purified by vacuum filtration through 0.45 μm regenerated cellulose membrane filters (Agilent, Böblingen, Germany). The compounds were separated on a C18 Bakerbond-BDC analytical column (250 mm×4.6 mm; 5 μm) (Avantor performance materials, Deventer, The Netherlands). Data were acquired with Agilent ChemStation software. Statistical analysis was performed using Microsoft Excel software.

The SPE procedure was performed using a Visiprep-DL Vacuum Manifold, 12-port model from Supelco (Bellefonte, USA) coupled to a vacuum pump from KNF Neuberger (Freiburg, Germany). Oasis HLB cartridges (30 mg, 1 mL, particle size 30 μm) were purchased from Waters (Milford, MA, USA).

Chromatographic conditions

The mobile phase was 30:70 (v/v) acetonitrile–10 mM sodium dihydrogen phosphate (pH 7.0 adjusted with 1 M sodium hydroxide). The flow rate was 1.5 mL min^{-1} and the column temperature was set at 35 °C. Detection was performed at 210 nm.

Human plasma samples

Patients and healthy volunteers who donated plasma samples gave written informed consent, and all investigations were approved by the Ethical committee (Faculty of Medicine, University of Niš, Serbia). Blank plasma was obtained from ten different healthy volunteers. Plasma samples from patients were obtained from Clinic of Neurology (University Clinical Centre Niš, Serbia). Blood samples were collected into vacutainers containing EDTA-Na, and separated by centrifugation at 3000 g for 10 min. All samples were stored at –80 °C before analysis.

Standard solutions, calibration standards and quality control samples

Four standard stock solutions of CBZ, CBZ-E, CBZ-DIOH and IS were prepared in acetonitrile at a concentration of 10 mg mL^{-1}. The standard working solutions of CBZ, CBZ-E, CBZ-DIOH and IS contained 1 mg mL^{-1} in the mobile phase. Standard stock solutions were stored at –20°C and standard working solutions were stored at 4–8 °C for 1 month. No stability related problems were encountered during this period.

Each analyte was added separately for the preparation of standard curve samples. Volumes of 0.1, 0.3, 2.5, 5, 7.5, 10 and 12.5 μL of 1 mg mL^{-1} CBZ, CBZ-E and CBZ-DIOH standard working solutions were transferred to seven Eppendorf tubes containing 0.5 mL of blank human plasma. Five μL of 1 mg mL^{-1} IS standard working solution was added to each of the Eppendorf tubes. After dilution and the SPE procedure 0.2, 0.6, 5, 10, 15, 20 and 25 μg mL^{-1} of CBZ, CBZ-E and CBZ-DIOH were obtained in the eluates. Concentration of IS was 10 μg mL^{-1}. The zero plasma samples were prepared by adding IS to drug-free human plasma to yield a final concentration of 10 μg mL^{-1}.

The quality control (QC) plasma samples were prepared to final concentrations of 0.6 (low), 10 (medium) and 20 μg mL^{-1} (high) of all the investigated compounds. The concentra-

tion of IS was 10 μg mL^{-1}. QC (medium) samples were used for the optimization of the SPE procedure.

QC samples were prepared daily, and parts of the low and high QC samples were stored at –80 °C to study their stability after three freeze–thaw cycles and long-term stability.

Solid phase extraction procedure

Phosphate buffer (10 mM of sodium dihydrogen phosphate, pH 7.0) was added to the prepared samples (calibration standards, QC samples) to a volume of 1 mL, which was followed by brief vortex mixing.

The flow rate during the SPE procedure was maintained at about 1.0 mL min^{-1}. The SPE cartridges were conditioned with 1 mL of methanol, and 1 ml of the phosphate buffer (10 mM; pH 7.0). The diluted plasma samples (1 mL) were loaded onto the cartridges. A wash step was performed with 1 mL of phosphate buffer (10 mM; pH 7.0) and subsequently 1 mL of methanol – 10 mM phosphate buffer, pH 7.0 (20:80, v/v). The cartridges were then dried under vacuum (–40 kPa) for 30 s. Finally, the analytes and IS were eluted with 0.5 mL of acetonitrile – 10 mM phosphate buffer, pH 7.0 (60:40, v/v). A 10 μL volume of the eluate was injected into the HPLC system for analysis.

RESULTS AND DISCUSSION

Development of the HPLC method

At the beginning of the investigation, log *D* values of the compounds were calculated using MarvinSketch software.[25] The log *D* values of CBZ-DIOH, CBZ-E and CBZ were 0.81, 1.97 and 2.77, respectively (Table I). The log *D* values were pH independent except for CBZ-DIOH, the ionic form of which appeared at pH values higher than 9.80. Therefore, the log *D* values of CBZ-DIOH, CBZ-E and CBZ are equivalent to their log *P* values at pH values lower than 9.80. Due to the lipophilic nature of CBZ-E and CBZ, a C18 column package was chosen. The following columns were investigated: Bakerbond-BDC C18 (150 mm×4.6 mm; 5 μm), Bakerbond-BDC C18 (250 mm×4.6 mm; 5 μm) and Symmetry C18 (150 mm×4.6 mm; 5 μm). The Bakerbond-BDC C18 analytical columns are better than the classical C18 columns owing to improved peak symmetry, lower back pressure and a longer column lifetime. By using Bakerbond-BDC C18 (150 mm×4.6 mm; 5μm) it was not possible to adequately separate the interfering plasma compounds from the analytes (CBZ, CBZ-E and CBZ-DIOH). Therefore, a longer column Bakerbond-BDC C18 (250 mm×4.6 mm; 5μm) was used. As a result, the analytes were successfully separated from the plasma compounds.

TABLE I. pK_a and log *D* values of the investigated compounds

Parameter	CBZ-DIOH	CBZ-E	CBZ
log *D*	0.81	1.97	2.77
pK_a	12.80	–	–

When methanol was a constituent of a mobile phase, peak symmetry and resolution between the contiguous analytes were poor. Considering the absorption of methanol at 210 nm, acetonitrile was examined as a constituent of the mobile phase.

CBZ and CBZ-E are neutral analytes, and CBZ-DIOH is a weak acid (pK_a = = 12.80). The examined pH range during the development of a chromatographic method is usually lower than 11. Therefore, it could be concluded that the pH of the mobile phase would not influence the retention of the investigated compounds, which was confirmed by a few experiments (mixtures 30:70 (*v/v*) acetonitrile–the phosphate buffer (10 mM) were used with a flow rate of 1.5 mL min^{-1} and column temperature at 35 °C with the pH adjusted to 6.2, 7 and 8.2). Furthermore, it was decided to use a mobile phase with the pH adjusted to 7. This decision was based on the chromatographic behavior of IS in order to shorten its retention time and hence the total run time.

Three variables were left to be optimized: percentage of acetonitrile, flow rate and temperature of the column. Firstly, a mixture 40:60 (*v/v*) acetonitrile–water was used as the mobile phase at a flow rate of 1 mL min^{-1} and with the column temperature at 30 °C. Under these chromatographic conditions, very poor retention of CBZ-DIOH was achieved. Afterwards, the percentage of water was increased and a mixture 35:65 (v/v) acetonitrile–water was used as the mobile phase, while the other chromatographic conditions remained the same. The retention factor of CBZ-DIOH was 0.37, although it was noticed that the decrease in the percentage of acetonitrile led to an increase in the retention factors of all the investigated compounds. Subsequently, a mixture 35:65 (*v/v*) acetonitrile– phosphate buffer (10 mM; pH 7.0) was used as the mobile phase at a flow rate of 1 mL min^{-1} and a column temperature of 30 °C. Addition of the phosphate buffer resulted in a better symmetry of the analyte peaks. Although the critical resolution of IS and CBZ-E peaks was improved, the retention factor of CBZ-DIOH was still less than 1. Therefore, the percentage of acetonitrile in mobile phase was decreased again and a mixture 30:70 (v/v) acetonitrile– phosphate buffer (10 mM; pH 7.0) was used at a flow rate of 1 mL min^{-1} and column temperature at 30 °C. Now the separation was successful and retention factor of CBZ-DIOH was 0.98, but chromatographic run lasted about 12 minutes. Hence, the flow rate was increased to 1.5 mL min^{-1} and the column temperature was increased to 35 °C. As a result, the total chromatographic run was shortened to about 8 min. Excellent peak shapes, good resolution between the contiguous peaks, and a number of theoretical plates of more than 17000 for all peaks were achieved. Retention factor of CBZ-DIOH was 1.02. Thus, these chromatographic conditions were chosen.

Development of the SPE procedure for sample pretreatment

Due to the different polarity of the neutral analytes (CBZ, CBZ-E and CBZ--DIOH), Oasis HLB cartridges were chosen since the polymeric sorbent retains both polar and non-polar compounds.[26]

As the analytes are neutral compounds and it was proven during development of the HPLC method that pH had no influence on the retention of the analytes, it was decided to use the buffer which was a constituent of the mobile phase (10 mM sodium dihydrogen phosphate, pH 7.0) for the conditioning of the cartridges, as well as for the wash and the elution steps. The selectivity was enhanced by tuning the ratio of the organic solvent to the phosphate buffer (10 mM; pH 7.0). Absolute recovery values of the investigated compounds were calculated for the optimization of the SPE procedure.

After load step of QC medium samples, the sorbent completely retained the analytes and IS. The first wash step was performed by passing 1 mL of the phosphate buffer (10 mM; pH 7.0), which did not remove any analyte or IS from the cartridge. For the second wash step, 1 mL of the mixtures 5:95, 10:90, 15:85, 20:80 and 30:70 (v/v) methanol–phosphate buffer (10 mM; pH 7.0) were investigated. The mixture 20:80 (v/v) methanol–phosphate buffer (10 mM; pH 7.0) was the highest percentage of methanol that did not remove any analyte or IS from the cartridge.

For the elution step, 0.5 mL of 30:70, 40:60, 50:50, 60:40 and 70:30 (v/v) acetonitrile–phosphate buffer (10 mM; pH 7.0) were investigated. The mixture 60:40 (v/v) acetonitrile–phosphate buffer (10 mM; pH 7.0) was the lowest percentage of acetonitrile which completely eluted the analytes and IS from the cartridge. The total time for sample preparation was shorter than 10 min.

Method validation

The new SPE-HPLC method was validated following FDA[23] and ICH[24] guidelines. The following validation characteristics were evaluated: selectivity, sensitivity, linearity, precision, accuracy, absolute recovery and stability.

The proposed method is selective since co-elution was not spotted at the retention times of CBZ, CBZ-E, CBZ-DIOH and IS from freshly prepared spiked samples at LLOQ levels compared to the blank plasma obtained from 10 healthy volunteers. The corresponding chromatogram of blank plasma sample is shown in Supplementary material to this paper.

The calibration curves showed good linearity over the investigated concentration range (0.2–25 μg mL^{-1} for all analytes). The obtained calibration curves were:

$$y = 0.1347x - 0.0118;\ r^2 = 0.9964 \text{ for CBZ-DIOH} \quad (1)$$

$$y = 0.1749x - 0.0725;\ r^2 = 0.9951 \text{ for CBZ-E} \quad (2)$$

and

$$y = 0.1426x - 0.0436;\ r^2 = 0.9976 \text{ for CBZ} \quad (3)$$

where y is peak area ratio, x is concentration of the compound and r is the correlation coefficient. The intercepts of the calibration curves were tested using the student's t-test. The following results were found for the standard deviation of

the slope (*Sa*), standard deviation of the intercept (*Sb*) and the confidence factor (t_α): *Sa* = 0.0051, *Sb* = 0.0716 and t_α = 0.1642 for CBZ-DIOH; *Sa* = 0.0078, *Sb* = = 0.1093 and t_α = 0.663 for CBZ-E; and *Sa* = 0.0044, *Sb* = 0.0615 and t_α = 0.7093 for CBZ. The deviation of the intercepts from zero were found to be insignificant (p = 0.05 and t_{tab} = 2.37). The corresponding chromatograms obtained from plasma sample spiked with IS, and plasma sample spiked with the analytes and IS in Supplementary material to this paper.

Limit of detection (*LOD*) and lower limit of quantification (*LLOQ*) values for all compounds were found to be 0.02 μg mL^{-1} and 0.2 μg mL^{-1}, respectively. The accuracy and precision were evaluated in five replicates at the *LLOQ* level. Accuracy is reported as recovery (*R* in %), precision as relative standard deviation (*RSD* in %) and the assessed values are given in Table II.

TABLE II. Intra-day precision and accuracy at *LLOQ*, low QC, medium QC, and high QC concentrations in plasma samples for CBZ-DIOH, CBZ-E and CBZ (n = 5)

Parameter	Nominal concentration in plasma, μg mL^{-1}			
	0.2	0.6	10	20
CBZ-DIOH				
Precision (*RSD* / %)	5.42	7.3	7.15	2.89
Accuracy (*R* / %)	102.63	92.72	108.50	103.27
Found concentration, μg mL^{-1}	0.205	0.556	10.85	20.65
CBZ-E				
Precision (*RSD* / %)	7.24	6.41	0.97	1.58
Accuracy (*R* / %)	114.41	98.60	98.95	98.30
Found concentration, μg mL^{-1}	0.229	0.592	9.895	19.659
CBZ				
Precision (*RSD* / %)	11.24	5.20	1.20	3.41
Accuracy (*R* / %)	89.05	102.77	98.71	103.87
Found concentration, μg mL^{-1}	0.178	0.617	9.87	20.77

After investigation of the intra-day and inter-day accuracy and precision at QC concentration levels, it was found that the obtained results for *RSD* (%) and recovery (*R*, %) were pursuant to FDA guidance[23] (precision of 20 % and accuracy of 80–120 % at the *LLOQ*; and precision of 15 % and accuracy of 85–115 % at the low QC, medium QC and high QC levels). The results are listed in Tables II and III.

To evaluate efficiency of the SPE procedure, the absolute recovery values were calculated. Spiked drug-free plasma samples at low QC, medium QC and high QC levels were diluted to 1 ml with the phosphate buffer and subjected to the SPE procedure. These samples were compared to blank plasma that had been extracted following the same SPE procedure and then spiked at the same concentration levels. The absolute recovery values as well as the estimated concentrations from human plasma for the investigated compounds are displayed in Table

IV. The absolute recovery values of all analytes did not appear to be dependent on concentration.

TABLE III. Inter-day precision and accuracy at *LLOQ*, low QC, medium QC, and high QC concentrations in plasma samples for CBZ-DIOH, CBZ-E and CBZ (n = 5)

Parameter	Nominal concentration in plasma, μg mL^{-1}		
	0.6	10	20
	CBZ-DIOH		
Precision (*RSD* / %)	7.96	4.11	0.81
Accuracy (*R* / %)	92.09	104.58	100.90
Found concentration, μg mL^{-1}	0.55	10.46	20.18
	CBZ-E		
Precision (*RSD* / %)	3.99	1.65	3.54
Accuracy (*R* / %)	93.34	98.23	104.04
Found concentration, μg mL^{-1}	0.56	9.82	20.81
	CBZ		
Precision (*RSD* / %)	1.14	2.16	5.42
Accuracy (*R* / %)	98.06	100.00	106.31
Found concentration, μg mL^{-1}	0.59	10.00	21.26

TABLE IV. Absolute recoveries of CBZ-DIOH, CBZ-E and CBZ from plasma samples (n = 5)

Parameter	Nominal concentration in plasma, μg mL^{-1}		
	0.6	10	20
	CBZ-DIOH		
Recovery, %	96.51	90.75	100.12
RSD / %	9.30	5.58	0.81
Found concentration, μg mL^{-1}	0.579	9.07	20.02
	CBZ-E		
Recovery, %	104.04	95.89	93.80
RSD / %	3.99	0.97	3.54
Found concentration, μg mL^{-1}	0.624	9.589	18.76
	CBZ		
Recovery, %	102.18	87.39	94.46
RSD / %	1.14	1.20	5.42
Found concentration, μg mL^{-1}	0.613	8.74	18.89

After performing the stability tests at low QC and high QC levels (short term, post-preparative, long-term stability and freeze–thaw cycles), the *RSD* values for the investigated compounds were below 8.39 %, while recovery values ranged from 90.84 to 112.35 %. Hence, the stability of the analytes was appropriate during all investigations. The results of the stability tests are given in Tables V–VIII.

Clinical application

Results of the assay of plasma samples obtained from nine epileptic patients under chronic treatment with CBZ are listed in Table IX. Along with CBZ, the

patients were co-administrated with amlodipine, bromazepam, dihydroergotoxine mesylate, aminophylline, fenoterol bromide, ipratropium bromide, beclomethasone dipropionate, prednisone, ramipril, losartan, acetylsalicylic acid, α-tocopherolacetate, nicergoline, lamotrigine, sodium valproate, topiramate, clonazepam, diazepam, lorazepam, phenytoin and ethosuximide. In all cases, no co-elution was observed at the retention times of the analytes and IS. To investigate additionally the selectivity of the method, plasma samples from healthy volunteers who had been administrated with the above-mentioned drugs were assayed and no interferences were registered.

On balance, the applicability of the method to routine analysis of plasma samples of epileptic patients under mono- and polytherapy was demonstrated. It allows for therapeutic drug monitoring of CBZ. However, the proposed method cannot be applied to analysis of plasma samples of patients co-administrated with CBZ and Phenobarbital, since it is used as an internal standard.

TABLE V. Results of short-term stability tests at low QC and high QC concentrations in plasma samples (n = 5)

Parameter	Nominal concentration in plasma, μg mL^{-1}	
	0.6	20
	CBZ-DIOH	
Recovery, %	99.71	105.49
RSD / %	2.93	4.44
Found concentration, μg mL^{-1}	0.598	21.10
	CBZ-E	
Recovery, %	90.84	104.26
RSD / %	8.39	1.01
Found concentration, μg mL^{-1}	0.545	20.85
	CBZ	
Recovery, %	91.82	110.22
RSD / %	3.81	1.43
Found concentration, μg mL^{-1}	0.551	22.04

TABLE VI. Results of the post-preparative stability test at low QC and high QC concentrations in plasma samples (n = 5)

Parameter	Nominal concentration in plasma, μg mL^{-1}	
	0.6	20
	CBZ-DIOH	
Recovery, %	101.56	99.77
RSD / %	6.81	0.64
Found concentration, μg mL^{-1}	0.609	19.95
	CBZ-E	
Recovery, %	96.18	99.82
RSD / %	5.57	1.28
Found concentration, μg mL^{-1}	0.577	19.96

TABLE VI. Continued

Parameter	Nominal concentration in plasma, μg mL^{-1}	
	0.6	20
	CBZ	
Recovery, %	96.42	100.66
RSD / %	5.47	2.52
Found concentration, μg mL^{-1}	0.579	20.132

TABLE VII. Results of the freeze–thaw stability test at low QC and high QC concentrations in plasma samples (n = 5)

Parameter	Nominal concentration in plasma, μg mL^{-1}	
	0.6	20
	CBZ-DIOH	
Recovery, %	112.35	102.66
RSD / %	2.20	1.36
Found concentration, μg mL^{-1}	0.674	20.53
	CBZ-E	
Recovery, %	102.40	102.66
RSD / %	7.29	1.69
Found concentration, μg mL^{-1}	0.614	20.53
	CBZ	
Recovery, %	105.42	103.63
RSD / %	2.64	1.22
Found concentration, μg mL^{-1}	0.63	20.73

TABLE VIII. Results of the long-term stability test at low QC and high QC concentrations in plasma samples (n = 5)

Parameter	Nominal concentration in plasma, μg mL^{-1}	
	0.6	20
	CBZ-DIOH	
Recovery, %	110.18	99.71
RSD / %	2.39	5.96
Found concentration, μg mL^{-1}	0.661	19.94
	CBZ-E	
Recovery, %	102.31	101.06
RSD / %	3.60	5.23
Found concentration, μg mL^{-1}	0.614	20.21
	CBZ	
Recovery, %	109.32	97.09
RSD / %	2.02	4.39
Found concentration, μg mL^{-1}	0.656	19.42

TABLE IX. Results of the assay of plasma samples obtained from nine epileptic patients under treatment with CBZ (n = 5)

Patient	Hours from last dosage	CBZ dosage, mg day^{-1}	(Mean concentration[a] ± *SD*[b]) / μg mL^{-1}		
			CBZ	CBZ-E	CBZ-DIOH
S.A	10.5	400	6.47±0.03	1.36±0.06	1.06±0.01
R.J.	10	600	8.74±0.05	2.06±0.02	3.06±0.04
M.L.	1	200	4.72±0.03	1.34±0.02	0.88±0.03
I.I.	9	400	7.12±0.07	1.44±0.03	1.40±0.06
A.D.	12	600	11.95±0.12	2.24±0.08	4.53±0.07
B.V.	13	1200	16.91±0.11	3.99±0.05	6.84±0.06
I.S	4	600	5.43±0.05	1.48±0.03	1.75±0.03
P.B.	3	400	8.7±0.07	1.69±0.04	1.48±0.09
T.R.	10.5	600	ND[c]	ND	ND

[a]Mean concentration in plasma, μg mL^{-1}; [b]standard deviation, μg mL^{-1}; [c]not determined, concentration was below the *LLOQ*

CONCLUSIONS

The chromatographic behavior of the investigated compounds was examined. As a result, an SPE-HPLC method was developed and validated. It showed satisfactory precision and accuracy with *RSD* values in the range from 0.81 to 11.24 % and recovery (*R*, %) values from 89.05 to 114.41 %. The linearity of the method was adequate in the range 0.20–25 μg mL^{-1} with a correlation coefficient higher than 0.9951 for the investigated compounds. The efficiency of the extraction procedure was established with the assistance of absolute recovery values, which were calculated to be from 87.39 to 104.04 %. During analysis of plasma samples obtained from patients, no interferences from endogenous compounds and co-administered drugs were found. Therefore, the developed chromatographic method was shown to be suitable for the simultaneous determination of CBZ and its metabolites CBZ-E and CBZ-DIOH in the plasma of epileptic patients. The short chromatographic run time and the rapid SPE procedure are important advantages of the proposed SPE-HPLC method for its routine application. In conclusion, the applicability of the method to therapeutic drug monitoring of patients under chronic treatment with CBZ was proved.

SUPPLEMENTARY MATERIAL

Representative chromatograms of blank plasma, blank plasma spiked with internal standard, blank plasma spiked with CBZ, CBZ-E, CBZ-DIOH and internal standard and plasma sample of an epileptic patient after receiving an oral dose of CBZ are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

РАЗВОЈ И ВАЛИДАЦИЈА SPE-HPLC МЕТОДЕ ЗА ОДРЕЂИВАЊЕ КАРБАМАЗЕПИНА И МЕТАБОЛИТА КАРБАМАЗЕПИН ЕПОКСИДА И КАРБАМАЗЕПИН *trans*-ДИОЛА У ПЛАЗМИ

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SPE-HPLC метода је развијена и валидирана у циљу брзог анализирања карбамазепина и метаболита карбамазепин епоксида и карбамазепин *trans*-диола у хуманој плазми. C18 Bakerbond-BDC аналитичка колона (250 mm×4,6 mm; 5 μm) је коришћена ради извођења анализе. Оптимални услови за хроматографско раздвајање су мобилна фаза ацетонитрил – 10 mM фосфатни пуфер, pH 7,0 (30:70, v/v), проток од 1,5 ml min^{-1}, температура 35 °C и детекција на 210 nm. Укупно трајање хроматографског рана износи око 8 min. SPE процедура за екстракцију аналита из узорака плазме је развијена уз коришћење Oasis HLB кетрица након чега се елуат инјектује у HPLC систем ради анализирања. Затим је извршена валидација SPE-HPLC методе. Линеарност је потврђена у концентрационом опсегу 0,2–25 μg/ml за карбамазепин, карбамазепин епоксид и карбамазепин транс-диол са вредношћу корелационих коефицијената вишом од 0,995. Прецизност методе у току једног и у току више дана је добра са релативном стандардном девијацијом нижом од 7,96 %, док тачност методе обухвата вредности у опсегу од 92,09 до 108,5 % за све аналите. На крају је метода успешно примењена у циљу анализирања узорака плазме пацијената оболелих од епилепсије на монотерапији и политерапији.

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